

The interference of piperidinopropionaphthone hydrochloride in mammalian type I and type II DNA topoisomerase reactions

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ABSTRACT

Majority of anti-cancer drugs were shown to exert their activities by interfering with DNA topoisomerase reactions. Since the identification of Camptothecin as the topoisomerase I targeting compound, these enzymes are widely utilized in biological assays to assess the pharmaceutical significance of the synthetic and natural agents. Because a considerable number of compounds were shown to have cytostatic activities via blocking topoisomerase reactions, we aimed to identify if the previously-reported physiological activities of acetonaphthones involves the interference with topoisomerase reactions. We covered topoisomerase activity and cytostatic activity evaluation of piperidinopropionaphthone hydrochloride type Mannich base (MB) to compare its bioactivities to the starting propionaphthone in order to assess the contribution of aminomethyl moiety of the compound on its bioactivity. MB was synthesized and

characterized in our laboratory. Supercoiled plasmid relaxation and decatenation assays were carried out to evaluate their biological activities in mammalian DNA topoisomerases. We also assayed the cytostatic activities using HeLa, MCF7 and A431 cell lines. Our data showed a considerable inhibition of MB on type I and type II DNA topoisomerases without a correlation to cytostatic assays. MB exerted a modest activity against the proliferation of MCF7 cells with an IC_{50} value of 27.62 μ M. The presence of MB inhibited topo II decatenation activity as well. Results offer no direct explanation for the contradictory effects on the DNA topoisomerases and the proliferation of cancer cells *in vitro*. Our results are discussed in relation to potential significance of aminomethyl group of Mannich base in the course of drug-development studies.

Key words: Mannich base, Anti-cancer drugs, Decatenation, Topoisomerase I, Topoisomerase II

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1. INTRODUCTION

A widely-applied approach in designing lead compounds is molecular combination of two potentially anticancer unites within a same molecule. Using this approach, we carried out the synthesis, structure elucidation and biological activity evaluation of an acetonaphthone (AN) derivative, piperidinopropionaphthone hydrochloride (Mannich base; MB), synthesized with a naphthalene ring system having intercalator property and a Mannich base connected to naphthalene ring at position 2 as alkylating agent. Acetonaphthone derivatives were previously reported to exert antispasmodic (1) and ganglioplegic (2) activities as well as partial inhibition on intracellular kinases (3). Initially, Mannich bases were synthesized as prodrugs of α , β -unsaturated ketones since deamination of Mannich bases *in vivo* and under simulated *in vitro* conditions have been documented (4). The use of Mannich bases in cancer therapy as cytostatic has been reported by several researchers (5 - 7). α , β -unsaturated ketones are important in cancer chemotherapy because these compounds are thiol

alkylators with little or no affinity for amino groups (8, 9). Mannich bases and the related α , β -unsaturated ketones are structurally different from today anticancer drugs. Thus they may be of use in treating neoplasms which have become resistant to current drugs. For example, increased GSH levels have been found in different tumor cell lines which have become resistant to the alkylating agent melphalan (10, 11) since thiols may be more abundant just prior to and during mitosis (12) and hence be more susceptible to alkylation. In fact, the concentration of glutathione (GSH) in cells is rapidly increased just prior to cell division (13). Besides Mannich bases provide improved aqueous solubility and hydrolytic stability of drugs like Doxorubicin.

Over the past several years, great majority anti-cancer drugs of synthetic and natural origin were shown to exert their activities by inhibiting DNA topoisomerases (14 - 20) as well as interfering in DNA-protein interactions (21). DNA topoisomerases are essential enzymes functioning in many genetic processes, by making transient breakages at nucleic acid backbone and rejoining of DNA strands (22). Transient breakages allow rotation of the DNA double helix around the intact phosphodiester bonds opposite to the enzyme-mediated DNA cleavages. Once the DNA is relaxed, the enzyme readily relegates the break and restores intact duplex DNA (15). These enzymes are classified as type I and type II topoisomerases according to their reaction mechanisms; Type I topoisomerases make a single-stranded break in a DNA duplex, mediate passage of the intact strand through the break, and then reseal it. Type II topoisomerases, on the other hand, create transient breaks in both strands of a duplex, pass an intact DNA segment through the break and then reseal the cleavage site (22, 23). Because DNA topoisomerases are important cellular targets of several compounds with or without intercalative properties, we tested MB and its parental compound, AN, in DNA topoisomerase reactions. We employed supercoiled plasmid relaxation and decatenation reactions using type I (topo I) and type (topo II) enzymes, respectively, as well as frequently utilized human adherent cell lines (HeLa, MCF7 and A431) to investigate the antiproliferative potency of the newly synthesized substances.

2. MATERIALS AND METHODS

2.1. Materials

All starting materials and reagents were high-grade commercial products obtained from Merck and Riedel. Human cancer cell lines HeLa (cervix adenocarcinoma) MCF7 (breast adenocarcinoma) and A431 (skin epidermoid

carcinoma) were purchased from ECACC (Salisbury, UK). The cells were cultivated in minimal essential medium supplemented with 10% fetal bovine serum, 1% non-essential amino acids and antibiotic-antimycotic mixture. The thermostat contained a humidified atmosphere of 5% CO₂ at 37 °C.

2.2. Chemistry

3-Piperidinopropio-2-naphthone hydrochloride (MB) was synthesized according to Mannich reaction conditions (24). The structure of the compound was confirmed by spectroscopic methods and elemental analyze (25). Melting points were determined with Buchi 510 capillary melting point apparatus and uncorrected. The IR spectra of compounds were monitored as potassium bromide pellets on a Jasco FT/IR-430 spectrometer. NMR spectra were recorded on a Varian AS 400 Mercury Plus NMR Spectrometer. Chemical shifts were measured in ppm (δ). Mass spectra were performed with Waters 2695 Alliance Micromass ZQ LC-MS Spectrometer Electro Spray Ionization (ESI). Elemental analyze were performed on a Thermo Finnigan EA 1112 C, H, N analyzer. UV spectra were taken on a spectrometer in methanol solution (UV-160, Shimadzu, Kyoto, Japan). Analytical thin-layer chromatography (TLC) was run on Merck silica gel plates (Kieselgel 60F₂₅₄) with detection by UV light.

2.3. Bioactivity experiments

2.3.1. Topoisomerase I supercoiled plasmid relaxation assays

Supercoiled plasmid relaxation assays were carried out as described (26). Briefly, 20 μ L of reaction mixture contained 500 ng of supercoiled (sc) plasmid DNA in the present or absence of the test compounds, one unit of calf thymus topo I (TAKARA, Otsu-Shiga, Japan), corresponding to the activity removing the supercoils from 500 ng of sc plasmid substrate at 37°C in the reaction buffer [35 mM Tris-HCl (pH 8.0), 72 mM KCl, 5 mM MgCl₂, 5 mM DTT, 5 mM spermidine, and 0.1% bovine serum albumin]. Reactions were carried out 30 minutes and terminated by the addition stop buffer (5% sarkosyl, 0.0025% bromophenol blue, 25% glycerol). Relaxation products were separated on 1% agarose gels in TBE buffer (45 mM Tris-Borate and 1mM EDTA, pH 8.0) in a horizontal electrophoresis apparatus (5V/cm) (Thermo EC250) and photographed under UV light after staining in ethidium bromide (Etd-Br) solution (0.5 μ g/mL). The quantitative evaluation of relative supercoiled and relaxed bands under UV light was carried out as described (27). DNA bands were quantified from gel

photo images using BioRad Multianalyst (ver. 1.1). Average band intensities were calculated from three independent reactions.

2.3.2. Topoisomerase II decatenation assays

The decatenation assay is a specific assay for topo II activity and based on detection of kinetoplast DNA, the mitochondrial DNA of *Crithidia fasciculata* found as a catenated network of DNA rings, the majority of which are 2.5 KB monomers (28). The assays were carried out using 0.2 µg kinetoplast DNA (K-DNA) substrate in a final volume of 20 µl in topo II reaction buffer (50 mM Tris-Cl, pH 8.0, 120 mM KCl, 10 mM MgCl₂, 0.5 mM ATP, 0.5 mM dithiothreitol). Reactions were terminated with stop buffer (5% sarkosyl, 0.0025% bromophenol blue, 25% glycerol) and electrophoretic analyses of K-DNA were performed using standard agarose gel containing 0.5 µg Etd-Br/mL. Resolution of decatenated DNA products were monitored as stated above. Etoposide, a well-known topoisomerase II inhibitor was used as control (0.5 mM).

1.3.3. Cytostatic assays

Antiproliferative effects of the test compounds were measured *in vitro* on three human cell lines: HeLa (cervix adenocarcinoma), MCF7 (breast adenocarcinoma) and A431 (skin epidermoid carcinoma), by using the MTT assay (29). Briefly, near-confluent cancer cells (5000/well) were seeded onto a 96-well microplate and attached to the bottom of the well overnight. On the second day, 200 µL of new medium containing the test substances was added. After incubation for 72 h at 37 °C in humidified air with 5% CO₂, the living cells were assayed by the addition of 20 µL of 5 mg/mL MTT solution. MTT was converted by intact mitochondrial reductase and precipitated as blue crystals during a 4 h contact period. The medium was then removed, and the precipitated crystals were dissolved in 100 µL of DMSO during a 60 min period of shaking at 25 °C. Finally, the reduced MTT was assayed at 545 nm, using a microplate reader; wells with untreated cells were utilized as controls. All *in vitro* experiments were carried out on two microplates with at least five parallel wells. Camptothecin was used as positive control. Stock solutions of the tested substances (10 mM) were prepared with DMSO. The highest DMSO concentration (0.3%) of the medium did not have any significant effect on the cell proliferation. Their antiproliferative effects were determined in the concentration range 0.1–30 µM, the dose-response curves were fitted by means of GraphPad Prism 4 (GraphPad Software, San Diego, CA, USA) and the IC₅₀ values were calculated.

3. RESULTS AND DISCUSSION

Reaction of piperidine hydrochloride with paraformaldehyde and acetonephthone (AN) led to the formation of the corresponding Mannich base (MB). The final compound has been reported for the first time in the literature with full spectral data (25).

We used plasmid DNA relaxation and DNA catenation assays in estimating biological activity. The former assay relies on the ability of topo I to relax sc DNA while the latter assay takes advantage a topo II-specific decatenation of minicircle DNA substrates. We monitored the interference by either compound according to the migration of substrate DNA molecules on agarose gel in the presence or absence of Etd-Br. A representative supercoil relaxation assay using 10 mg/mL of parental compound (AN) and its derivative (MB) is given in Fig. 1. As seen in Fig. 1, sc plasmid substrate, pBR322 (Fig. 1, lane 1) is relaxed by topo I (Fig. 1, lane 2) in the absence of the test compounds. The supercoil relaxing activity of topo I was not influenced by 10% DMSO, used in dissolving the test compounds (Fig. 1, lane 3). However, the reaction was profoundly affected in the presence of 1 mg/mL MB (Fig. 1, lane 5) while the parental compound, AN was not effective on topo I reaction (Fig. 1, lane 4).

The results we obtained by using a single concentration of the compounds led us to estimate the minimum concentration of MB required for interference on topo I activity upon serial dilutions (Fig. 2). As expected, there was no interference manifested by AN throughout the concentrations we employed (Fig. 2A, lanes 1 and 5). The

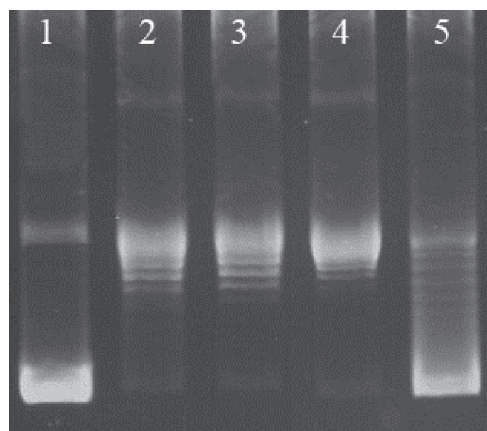


Fig. 1. The effect of 2-Acetonaphthone (AN) and its Mannich derivative, piperidinopropionaphthone hydrochloride (MB) on mammalian DNA topoisomerase I activity. Lane 1, sc plasmid substrate, pBR322; lane 2, pBR322 with 1 u topo I; lane 3, same as lane 2 in the presence of 10% DMSO; lane 4 and 5; same as lane 2 in the presence of 1 mg/mL MB and 1 mg/mL AN, respectively (see “Materials and Methods” for the details).

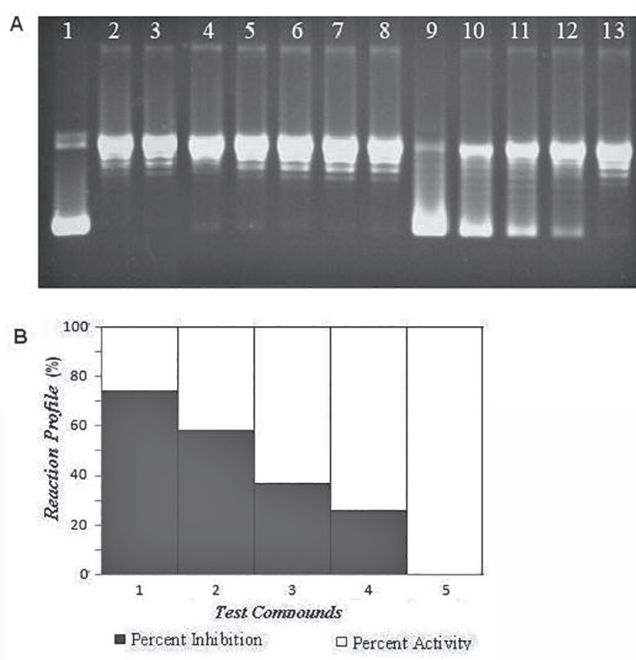


Fig. 2. A representative agarose gel photograph of supercoil relaxation in the presence of varying concentrations of test compounds and its quantitative assessment. **A.** Lane 1, pBR322; lane 2, pBR322 with 1 u of DNA topoisomerase I; lane 3, same as lane 2 in 10% DMSO; lanes 4-8, same as lane 2 plus 10 mg/mL, 5 mg/mL, 2.5 mg/mL, 1 mg/mL and 0.5 mg/mL of AN, respectively; lanes 9-13, same as as lane 2 plus MB in the same concentration order of lanes 4-8. **B.** Quantitative assessment of the reaction profile obtained with MB. The concentrations of MB from 1 to 5 correspond to 10 mg/mL, 5 mg/mL, 2.5 mg/mL, 1 mg/mL and 0.5 mg/mL, respectively.

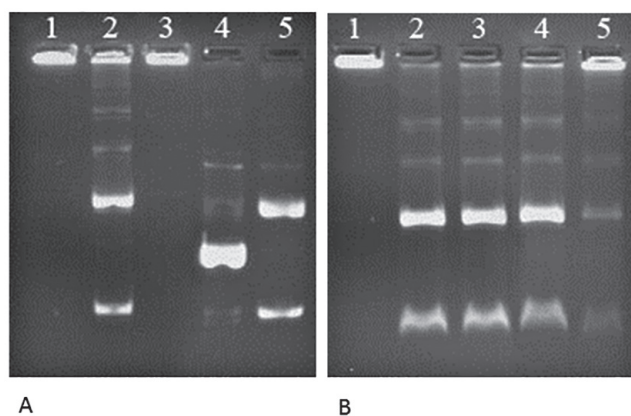


Fig. 3. Principle of topoisomerase II decatenation and a representative decatenation assay using the test compounds. **A.** A representation of topoisomerase II decatenation assay. Lane 1, catenated DNA; lane 2, catenated DNA in the presence of 1 u Topoisomerase II; lane 3, same as lane 2 in the presence of Etoposide; lane 4, linear DNA marker; lane 5, decatenated DNA marker. **B.** A representative Topoisomerase II decatenation assay. Lane 1, catenated DNA without topoisomerase II; lane 2, same as lane 2 in the presence of 1 u of Topoisomerase II; lane 3, same as lane 2 in the presence of 10% DMSO; lane 4-5, same as lane 2 in the presence of 1 mg/mL 2-Acetonaphthone and Piperidinopropionaphthone hydrochloride, respectively.

interference obtained by 10 mg/mL MB (Fig. 2A, lane 9) was gradually decreased upon dilution the test compound to 5 mg/mL and 2.5 mg/mL (Fig. 2A, lanes 10 and 11, respectively). The effect was neglectable upon dilution of MB to 1 mg/mL (Fig. 2A, lane 12) as it gave rise to a very faint sc DNA band with an accumulated slower-migrating relax DNA, which eventually disappeared at 0.5 mg/mL concentration (Fig. 2A, lane 13). This indicates that the effect of MB was concentration-dependent. Densitometric calculations of relative band intensities showed that the interference was by 74% at 10 mg/mL MB concentration and this value gradually decreased to 58%, 37% and 26% upon serial dilutions (Fig. 2B). We calculated a high IC_{50} value of 825 μ M for MB by considering residual nicked-circular DNA present in plasmid prep into account during quantitative evaluations (28).

We then monitored decatenation activity of topoisomerase II in the presence or absence of the test compounds (Fig. 3). The method takes advantage of inclusion of Etd-Br that separates open-circular (OC) monomers from covalently-closed circular (CCC) DNA. As seen in Fig. 3, the K-DNA was too large to enter the gel (Fig. 3A, lane 1), however, purified eukaryotic topoisomerase II yielded substantial monomeric DNA rings of two species, OC-DNA and CCC-DNA (Fig. 3A, lane 2) as they were positioned with the same banding patterns of linear (Fig. 3A, lane 4) and decatenated DNA markers (Fig. 3A, lane 5). The reaction was completely abolished in the presence of Etoposide (Fig. 3A, lane 3). A representative decatenation assay using equal concentration of AN and MB is given in Fig. 3B. Compared to first two lanes, corresponding to catenated DNA substrate and its decatenated products by the action of topo II, AN did not exert any detectable interference (Fig. 3B, lane 4) while the presence of MB inhibited topo II decatenation (Fig. 3B, lane 5). The activity of topo II was not influenced by 10% DMSO (Fig. 3B, lane 3). The dilution of MB resulted in a diminished decatenation of the enzyme (data not shown). Because high IC_{50} values (>100 μ M) are not considered as conclusive from practical point of view as such high concentrations are rarely elicitable under *in vivo* conditions.

We next measured antiproliferative effects of the test compounds on three human cell lines: HeLa (cervix adenocarcinoma), MCF7 (breast adenocarcinoma) and A431 (skin epidermoid carcinoma), by using the MTT assay (29) (Fig. 4). AN exhibited no substantial cytostatic effect on the three utilized human cell lines up to 30 μ M. Its MB congener proved to be similarly ineffective on HeLa and A431 cells but exerted a modest activity against the

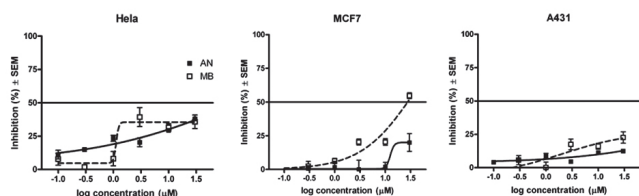


Fig. 4. The cell proliferation inhibiting effects of AN (■, solid line) and MB (□ dotted).

proliferation of MCF7 cells which could be characterized by the calculated IC_{50} value of 27.62 μ M (Fig. 4).

A relatively more profound bioactivity of final compound (MB) compared to starting ketone might be attributed to the α , β -unsaturated ketone liberated from the Mannich bases by deamination to undergo Michael type addition with DNA nucleophiles (30).

Taken together, the presented set of results offer no direct explanation for the contradictory effects on the DNA

topoisomerases and the proliferation of cancer cells *in vitro*. The IC_{50} we calculated in supercoiled plasmid relaxation reflects the nature of the assay. Indeed, IC_{50} value for Camptothecin, which is a well-known anti-cancer the compound, gave rise to a higher IC_{50} value than its expected *in vitro* efficacy (18 and data not shown). On the other hand, the AN and MB were added to the medium and penetration through an intact cell membrane is a condition of their action in MTT assay. A close relationship between cytostatic effect and lipid solubility of a set of alkaloids has been published earlier (31). Therefore, it could be speculated that MB has a limited capacity to enter the cell and interact with the DNA which is reflected in its poor overall antiproliferative property.

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Piperidinopropiyonafton hidroklorür bileşiğinin tip I ve tip II memeli DNA topoizomeraz enzimleri üzerindeki etkisi

ÖZET

Antikanser ilaçların çoğunun hücrede sıklıkla bulunan DNA topoizomeraz reaksiyonları ile girişim yaparak etkinliklerini ortaya koyduğu gösterilmiştir. Nitekim kamptotesinin (camptothecin) topoizomeraz I enzimini hedefleyen bileşik olarak tanımlanmasından itibaren sentetik ve doğal bileşiklerin farmasötik önemini değerlendirmek üzere biyolojik çalışmalarda bu enzimlerden geniş ölçüde yararlanılmıştır. Önemli sayıda bileşiğin topoizomeraz reaksiyonları üzerinden sitotoksik etkinliğe sahip olmaları nedeniyle asetonafonun ve türevinin daha önce rapor edilen fizyolojik aktivitelerinin topoizomeraz reaksiyonlarını içerip içermediklerinin araştırılması hedeflenmiştir. Başlangıç molekülü olan asetonafonun (AN) biyoaktivitesi ile karşılaştırılmak üzere bileşikte bulunan aminometilin biyoaktiviteye etkisi, Mannich bazı türevi olan

piperidinopropiyonafton hidroklorür bileşiği (MB) ile topoizomeraz aktivite ve sitostatik aktivite ölçümleri kullanılarak değerlendirilmiştir. MB laboratuvarımızda sentezlenip karakterize edilmiştir. Memeli DNA topoizomerazları üzerindeki biyolojik aktivitelerini değerlendirmek üzere süpersarmal plazmid relaksasyon ve dekatensasyon deneyleri gerçekleştirilmiştir. Ayrıca HeLa, MCF7 ve A431 hücre hatlarında sitostatik aktivite araştırılmıştır. Verilerimize göre sitostatik değerlendirmelerden bağımsız olarak MB, tip I ve tip II DNA topoizomeraz üzerinde dikkate değer bir etki göstermiştir. MB, MCF7 hücrelerindeki proliferasyona karşı 27.62 μ M olan IC_{50} değeri ile ortalama bir etki göstermiştir. Bununla birlikte MB varlığı topoizomeraz II dekatensasyon aktivitesini etkilemiştir. *In vitro* koşullarda sonuçlarımız DNA topoizomerazlar ve kanser hücrelerinin proliferasyonu üzerindeki etkilerini direkt olarak açıklamamaktadır. Sonuçlarımız Mannich bazının ilaç geliştirme çalışmalarındaki potansiyel önemi üzerinden değerlendirilmiştir.

Anahtar Kelimeler: Mannich bazı, Antikanser ilaçlar, dekatensasyon, topoizomeraz I, topoizomeraz II

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